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The FLB-10 provirus represents a full-length molecular clone derived from SIVmac 251 simian immunodeficiency virus. The molecularly cloned virus is replication-competent in vitro and is cytopathic for CEMX174 lymphocytes and primary macaque peripheral blood lymphocytes. Complete nucleotide sequence reveals that the FLB-10 clone contains mutations compromising expression of the vpr, nef and env genes. Therefore, vpr and nef gene products are not necessary for the in vitro replication or cytopathic effect of SIVmac. Virus stocks were inoculated into several cynomolgus monkeys and one juvenile rhesus macaque. While none of the cynomolgus monkeys have developed an infection over one year post-inoculation, the injected macaque became infected and developed a fatal immunodeficiency syndrome. These results will stimulate further studies with larger numbers of animals to address whether vpr or nef reversions occur to allow in vivo SIV replication.

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FOREWORD

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Introduction

Simian immunodeficiency viruses are retroviruses isolated from captive and feral monkey populations. Although there are as yet no examples of disease induced by SIV in natural hosts, several biologically cloned SIVs induce AIDS - like disease in juvenile rhesus macaques. Recently, it has been observed that molecular clones of SIVmac and SIVpbj can give rise to viruses that are pathogenic in rhesus macaques. The SIVmac virus induces a chronic AIDS-like disease characterized by CD4-lymphocyte depletion whereas the SIVpbj induces an acute gastrointestinal disease involving the lymphoid cells of the gut. Other molecular clones of SIV replicate poorly in macaques and fail to induce disease. The genetic determinants of relative replication rate and pathogenicity of different SIV variants are presently unknown. These determinants may reside in the transcriptional regulatory functions of the viral long terminal repeat (LTR), in the composition of the viral regulatory genes (vif, vpx, vpr, tat, rev, and nef), or in features of the structural components of the virus.

To understand the molecular determinants of SIV pathogenicity, the following approach was utilized:

1. Establishment of conditions for efficient in vitro propagation of SIVmac 251.

2. Cloning of full-length proviruses from infected cells using novel cloning vectors.

3. Characterization of in vitro infectivity and cytopathogenicity of viruses derived by transfection of full-length proviral clones.

4. Complete nucleotide sequence determination of an infectious molecular clone of SIVmac251.

5. Determination of in vivo replication and pathogenesis of the virus derived from the infectious molecular clone.

Methods

Cell lines Jurkat, HUT78 and CEMX174 human T lymphocyte lines were propagated in RPMI 1640 plus 10 per cent fetal calf serum. The latter cell line is a human B-T cell hybrid supplied by James Hoxie at the University of Pennsylvania School of Medicine. The HUT78 cells producing a high titer of the SIVmac251 virus were supplied by Ron DesRosiers at the New England Primate Center.

Provirus cloning DNA was extracted from CEMX174 cells infected with SIVmac251 and digested prior to packaging in the phage arms of the EMBL3 bacteriophage. The library was constructed according to standard procedures and screened with an LTR fragment derived from an SIV subgenomic clone kindly provided by James Mullins at the Harvard School of Public Health. For subcloning phage inserts containing full-length SIV proviruses, the inserts were initially cloned into phasmids, bacteriophage vectors containing additional insert sequences that

allow plasmid maintenance in bacterial hosts. The SIV infectious provirus FLB-10 was propagated as a phasmid in E.coli in liquid culture and plasmid DNA was prepared. After digesting the phasmid DNA with restriction endonucleases that did not recognize the SIVmac proviral insert sequences, the plasmid insert containing the provirus was circularized by DNA ligase and used to transform E.coli. Ampicillin-resistant colonies were grown in small batches at room temperature to maintain the integrity of the provirus and to minimize recombination. Plasmid DNA prepared from bacteria grown in this manner yielded restriction maps consistent with the presence of full-length proviruses.

Generation of virus The bacteriophage recombinant proviral clones were tested for ability to generate replication-competent, cytopathic viruses in tissue culture. Methods for the efficient transfection of the CEMX174 cell line with high molecular weight DNA were tested. Electroporation of the DNA into the cells was found to be effective for DNA introduction. Approximately 30 micrograms of phage DNA containing full-length SIV proviruses was electroporated using the Bio-Rad electroporation system at 260V, 960 microfarads. Electroporated CEMX174 cells were incubated in phosphate-buffered saline at 0°C for 5 minutes prior to and 10 minutes following electroporation. SIVs derived by electroporation were propagated on CEMX174 and HUT78 cells and virus stocks of greater than 10^6 reverse transcriptase units per milliliter were generated. These stocks were used for intravenous inoculation of cynomolgus and juvenile rhesus monkeys at the Medical Research Council primate facility in the United Kingdom.

Provirus sequencing using phage DNA The Sanger method of dideoxynucleotide sequencing was modified to allow direct sequencing of double-stranded proviral DNA contained in the lamba phage vector. This allows rapid cloning and sequencing of many proviral clones to assess heterogeneity in a given virus population. The modifications to the standard Sanger method are:

1. An increased primer-to-template ratio is utilized;
2. Denaturation and primer-template annealing are performed simultaneously at 100°C rather than under alkaline conditions at 85°C.

These two changes allow an increased likelihood of primer annealing to the proper initiation site on the template rather that initiating non-specifically.

Provirus sequencing using plasmid DNA The majority of the FLB-10 proviral DNA was sequenced according to the following method. Oligonucleotide primers were prepared based on consensus sequences for available SIV isolates provided in the Los Alamos data bank. Approximately 85% of these primers were found to yield readable sequence information from the pFLB-10 provirus. An additional set of primers was prepared based on the sequence of cDNA clones derived from SIVmac 251 - infected cells. The latter primers were found to prime readable sequencing reactions using the pFLB-10 provirus in nearly 100% of the cases. In total, sequencing reactions with approximately 45 different primers were used to complete the sequence of the entire pFLB-10 provirus.

Sequencing reactions for double-stranded plasmids were carried out according to the following protocols based upon the method of Sanger et al. Briefly, template DNA was prepared by treatment of the pFLB10 plasmid with alkali to denature the DNA, followed by strand separation on denaturing acrylamide gels. The primer was annealed to the single-stranded template DNA

by heating to 65°C then slowly cooling the reaction to 35°C. The molar ratio of primer:template for most reactions ranged from 1:1 to 3:1. The annealed primer:template was mixed with the 6mM DTT, labelling mix and sequenase enzyme (Sequenase Kit, USB Corporation) and [^{35}S] - dATP, then incubated at room temperature for 2-5 minutes. The reactions were terminated by addition of ddATP, ddGTP, ddTTP and ddCTP, incubation at 37°C for 3-5 minutes, and addition of stop solution (Sequenase Kit, USB Corporation). Samples were loaded on simultaneous 4% and 8% acrylamide sequencing gels, or in some cases, on 6% gradient gels. Each sequencing reaction typically yielded from 400 to 550 base pairs of information. Each segment of the genome was sequenced using at least two sets of non-overlapping primers.

Results and Discussion The initial phase of the work on simian immunodeficiency virus (SIV) consisted of the establishment of in vitro tissue culture systems for propagating these viruses. Several cell lines were tested for ability to support SIV replication in vitro, including Jurkat, HUT78 and CEMX174. The CEMX174 cell line was found to be most efficient at supporting replication of SIVmac. Upon cocultivation of SIVmac-infected cells that were producing only low titers of virus with the CEMX174 cells, marked syncytium formation and release of high amounts of virion-associated reverse transcriptase into the medium were observed. Evidence for continuous high-level virus replication in the CEMX174 target cells was obtained by measuring virus envelope-specific cell surface fluorescence, reverse transcriptase, viral protein expression by radio-immunoprecipitation, and proviral DNA by Southern blotting. Budding, immature and mature virion particles were observed by electron microscopy. Cytopathic effects were observed in the CEMX174 cells infected with SIVmac and included syncytium formation and single cell cytolysis. The high level of SIVmac replication obtained in the CEMX174 cell line suggested that this line might provide a good source of DNA for the purpose of cloning an infectious SIV provirus. The DNA was extracted from CEMX174 cells that were infected with SIVmac and used to construct a genomic library in bacteriophage. The library was screened as described in Materials and Methods. By screening recombinant bacteriophage, ten full-length proviral clones of SIVmac were derived. Restriction endonuclease mapping of the ten clones revealed them to possess primarily common, and some unique, restriction sites.

One of the proviral clones, FLB-10, was found to generate replication competent SIV following electroporation into CEMX174. Viral proteins produced in the infected cells corresponded to those expected for SIVmac using serum from SIV-infected animals. The generated virus was observed to induce cytopathic changes in the CEMX174, including syncytium formation and single cell killing. The generated virus could be transmitted in a cell-free manner to uninfected HUT78 or CEMX174 cells.

The FLB-10 insert containing the infectious SIVmac provirus was subcloned into a plasmid. The maintenance of full-length SIV proviruses on plasmids has been hampered by poor growth of the host plasmid sequences and the tendency to sustain deletions, even in recA-hosts. Initial attempts to subclone the FLB-10 insert directly into a plasmid were unsuccessful due to the aforementioned problems. Subsequently, a two-step cloning procedure described in the Materials and Methods was developed that allowed successful propagation and manipulation of SIV proviruses on plasmids. SIVs derived from the infectious pFLB-10 cloned were propagated on CEMX174 and HUT78 cells and virus stocks used for intravenous inoculation of macaques and cynomolgus monkeys. At 14 months post-infection, none of four cynomolgus monkeys

inoculated appear to be infected as judged by the lack of appearance of anti-SIV antibodies by Western blotting or immunoprecipitation. However, the single macaque infected with 10^6 reverse transcriptase units intravenously died at 14 months post-infection of an immunodeficiency characterized by CD4-positive lymphoid depletion and opportunistic infection. The serum of the animal is in the process of being analyzed retrospectively for the presence of anti-SIV antibodies, and subgenomic fragments of the provirus will be amplified from peripheral blood lymphocytes by the polymerase chain reaction. The sequence of the 3' half of the FLB-10 provirus is shown in appendix 1 and is summarized in Figure 1. The nucleotide sequence of the FLB-10 clone is generally similar to that of other SIVmac251 isolates and is more similar to those isolates than to SIV's derived from mangabeys or African green monkeys. The major open reading frames in the FLB-10 provirus are as follows:

1. vpx

   The vpx protein is not necessary for SIV replication, but appears to stimulate virus replication through an unknown mechanism. The vpx open reading frame is present and a potential initiator methionine is evident at the 5' end of the reading frame. The FLB-10 vpx open reading frame could encode a protein of 113 amino acids, with a proline-rich segment near the carboxyl terminus. The vpx of FLB-10 differs from the consensus SIV sequence at one residue (62) M K.

2. vpr

   The vpr protein is not necessary for HIV-1 gene expression, but can stimulate HIV-1 replication through its effect as a promiscuous trans-activator of gene expression. Most SIVmac isolates have an open reading frame for vpr, with heterogeneity at the 3' end. The BK28 isolate encodes a 98 amino acid vpr product, while MM142 encodes a 102 amino acid vpr protein. The vpr open reading frame of the FLB-10 provirus has obviously lost the potential to encode a protein, since the methionine likely to be utilized for initiation, based on sequence similarity to other SIVmac provirus clones, is followed within eleven codons by a stop condon. Following the stop codon, the vpr open reading frame continues with strong sequence similarity to the vpr sequence of other SIV isolates, so it is likely that correction of the single stop codon will result in a full-length vpr product. This potential vpr product would be 98 amino acids long, similar in size to functional vpr proteins observed in the HIV-1 system. In addition, there are two vpr changes in FLB-10 differing from the consensus SIV sequence (47) 1 M and (77) C S.

3. tat and rev

   The FLB-10 tat and rev open reading frames are intact, as would be expected for an infectious proviral clone. Tat is a positive trans-activator of viral gene expression, while rev is a post-transcriptional regulator of structural protein expression. The tat gene of FLB-10 differs from consensus by only two changes (27) A R and (75) S C, while the FLB-10 rev gene demonstrates no changes from the SIV consensus sequence.

4. env

The FLB-10 envelope glycoproteins are similar to other SIVmac isolates in that a premature stop codon exists in the transmembrane glycoprotein. It appears that either one of a pair of CAG residues is converted to a TAG amber codon in various SIVmac isolates passaged in human cell lines. The FLB-10 clone has a TAG CAG sequence, similar to that seen in the SIVmac251 but unlike the other SIVmac isolates. In addition, the FLB-10 has a second premature stop codon that would result in a deletion of 18 carboxy-terminal amino acids.

5. nef

Although premature stop codons in HIV-I nef genes are common, most SIV molecular clones do not have obvious premature nef stop codons. Heterogeneity occurs in the carboxyl terminus of nef in different SIV isolates (from 248-299 amino acid residues). The FLB-10 nef gene, which otherwise could encode a 263 residue protein, contains at least two defects that render it non-functional. First, the nef initiator methionine codon (ATG) has been mutated to an ATA, encoding isoleucine. The next residue, a glycine, which is important for myristillic acid addition, has been preserved in the FLB-10 provirus. Second, a premature stop codon results in only a 94 amino acid product, even if the initiator methionine were present. This result explains the observation that deletions of the FLB-10 provirus in the nef region did not result in phenotypic differences in viral replication rate or in cytopathic effect.

These results suggest that neither vpr nor nef is required for SIV infection in tissue culture, an observation consistent with that reported for the human immunodeficiency virus type I. The ability of the FLB-10 molecular clone to generate a virus capable of replicating and causing disease in an inoculated macaque will need to be verified in larger numbers of juvenile macaques. Studies to amplify subgenomic segments of the in vivo replicating virus from the peripheral blood of the infected macaque are in progress. Of particular interest will be the status of the vpr, nef, and env open reading frames. If the stop codons or other mutations in these FLB-10 genes have reverted in the infected animal, this would strongly support the existence of an in vivo advantage to SIV for competence at expressing these gene products.

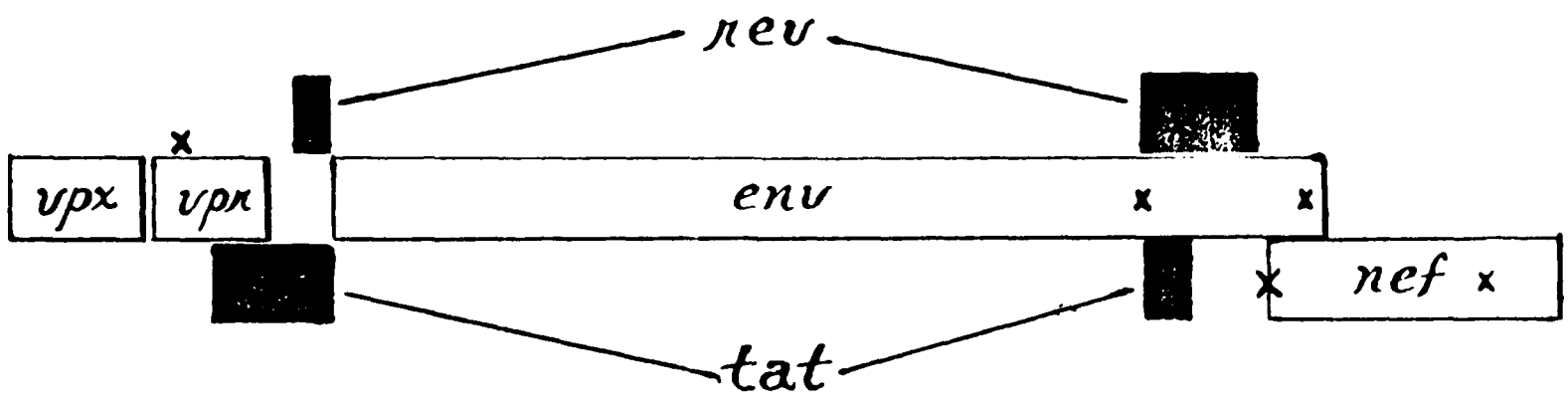
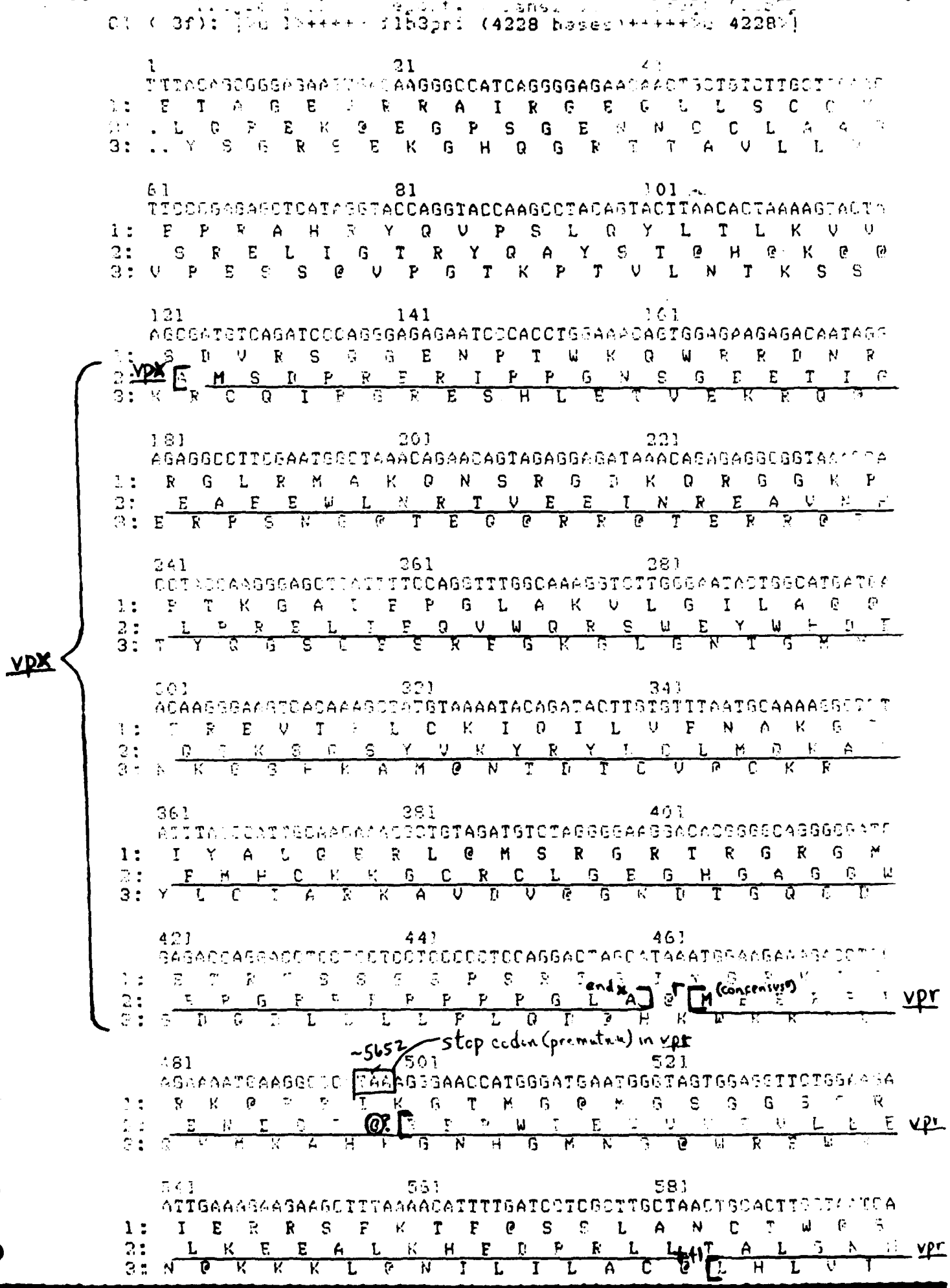
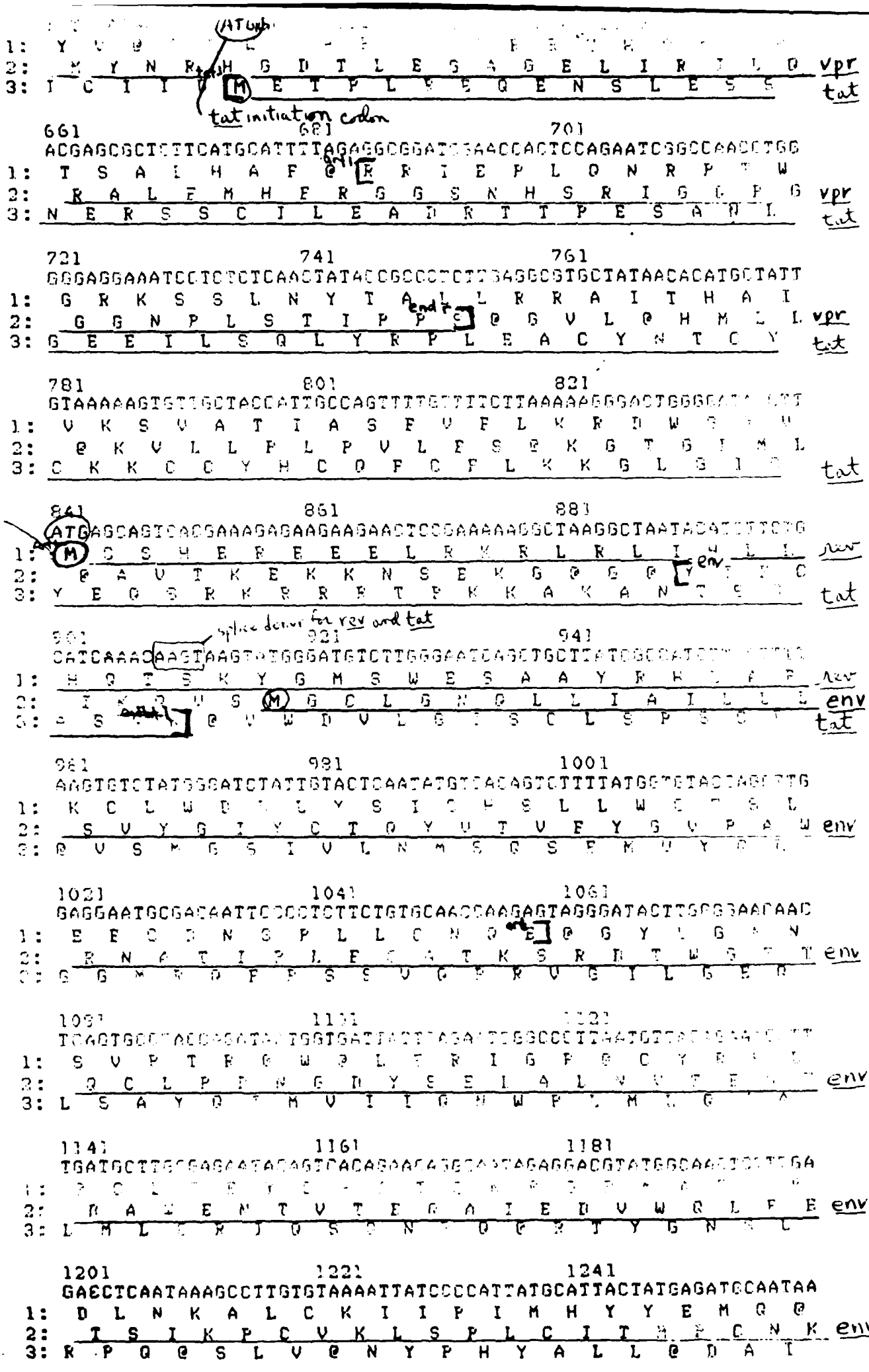


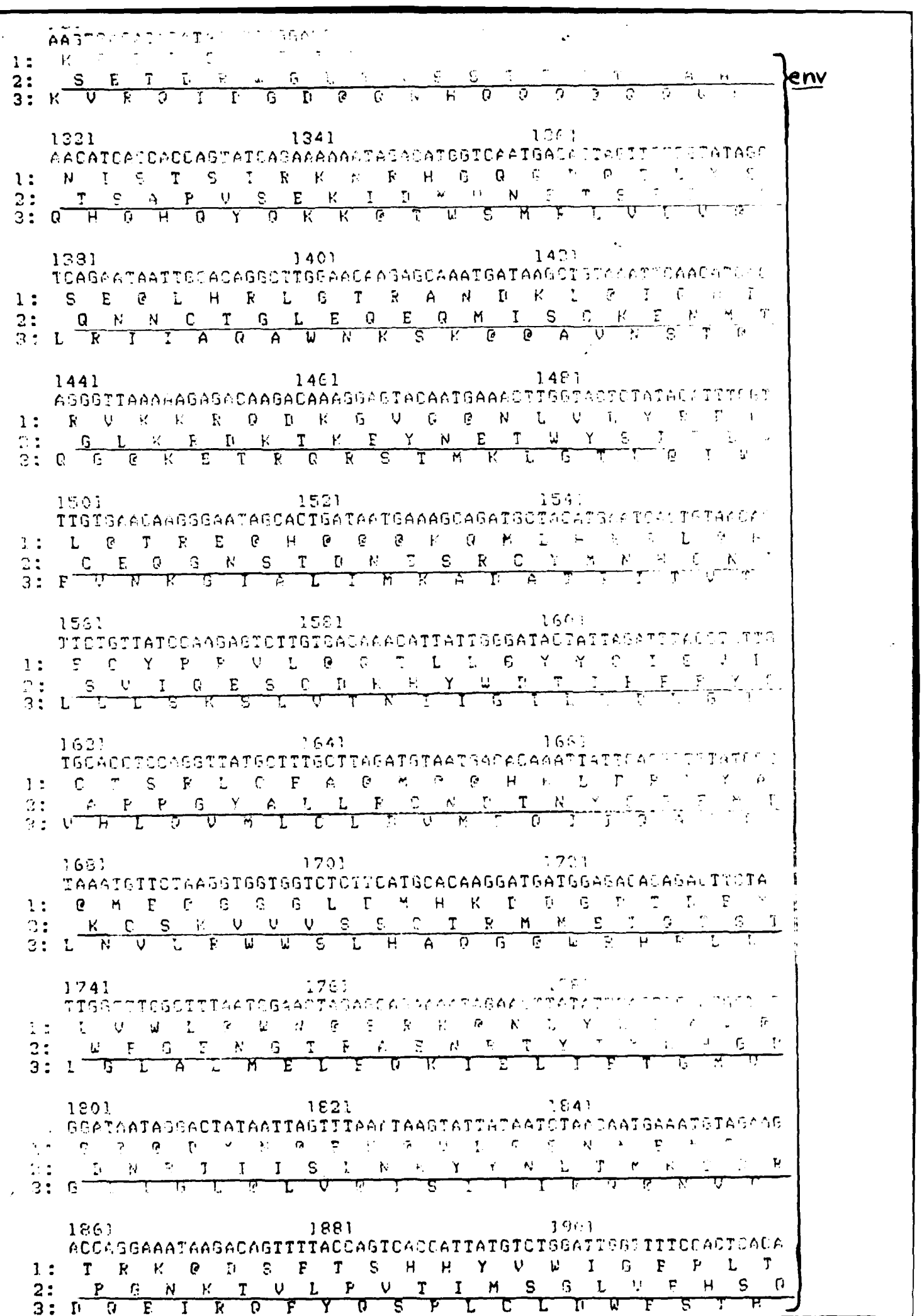
Figure 1. Schematic diagram of the FLB-10 3' half of the provirus. The vpx, vpr, tat, rev, env and nef genes of the FLB-10 SIV_{mac} 251 isolate are shown. The X's represent changes in the FLB-10 sequence that render the open reading frame products either unable to be synthesized or prematurely terminated. The dark boxes represent the two coding exons of the functional tat and rev genes of FLB-10.

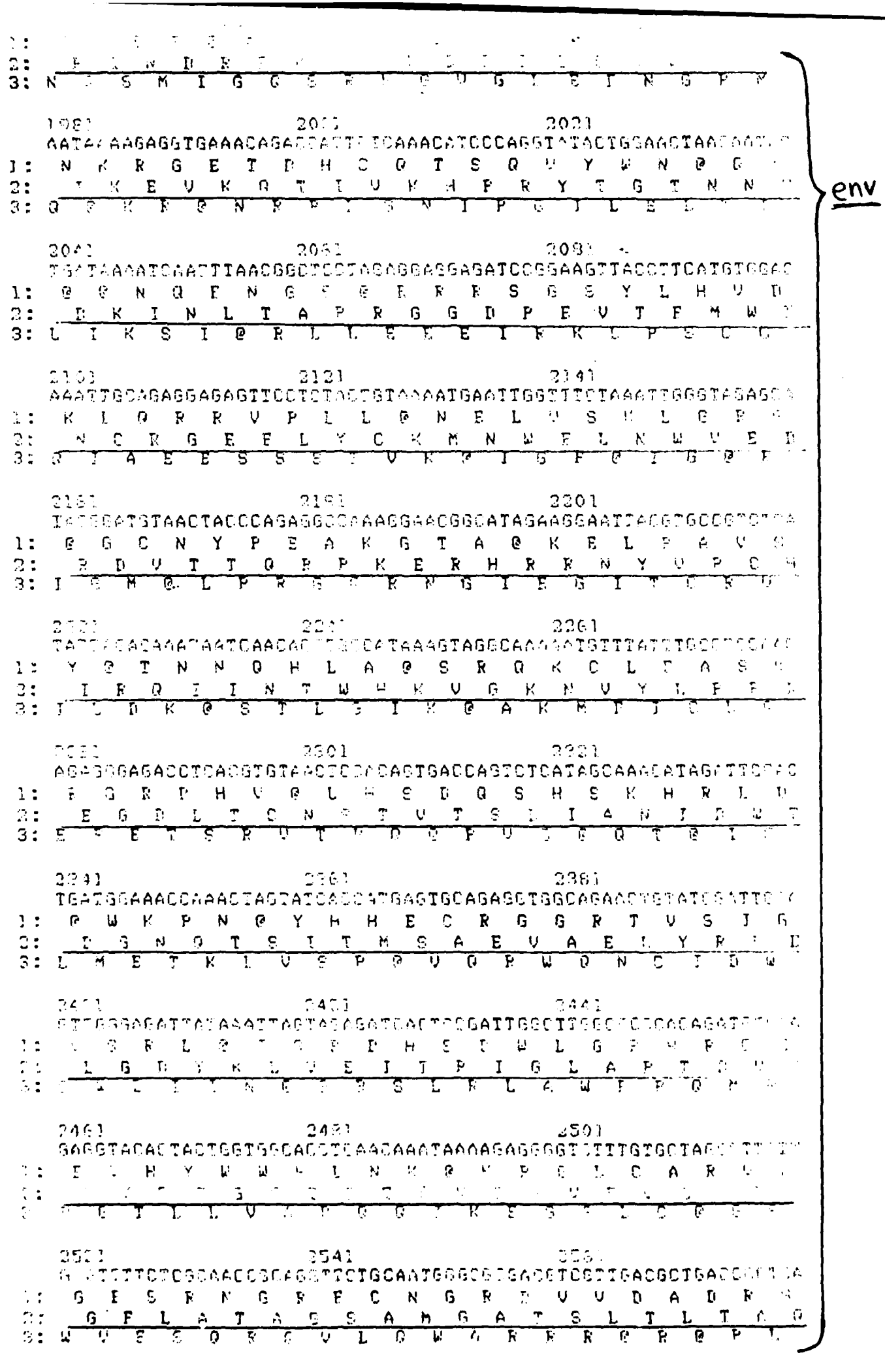
Appendix 1.

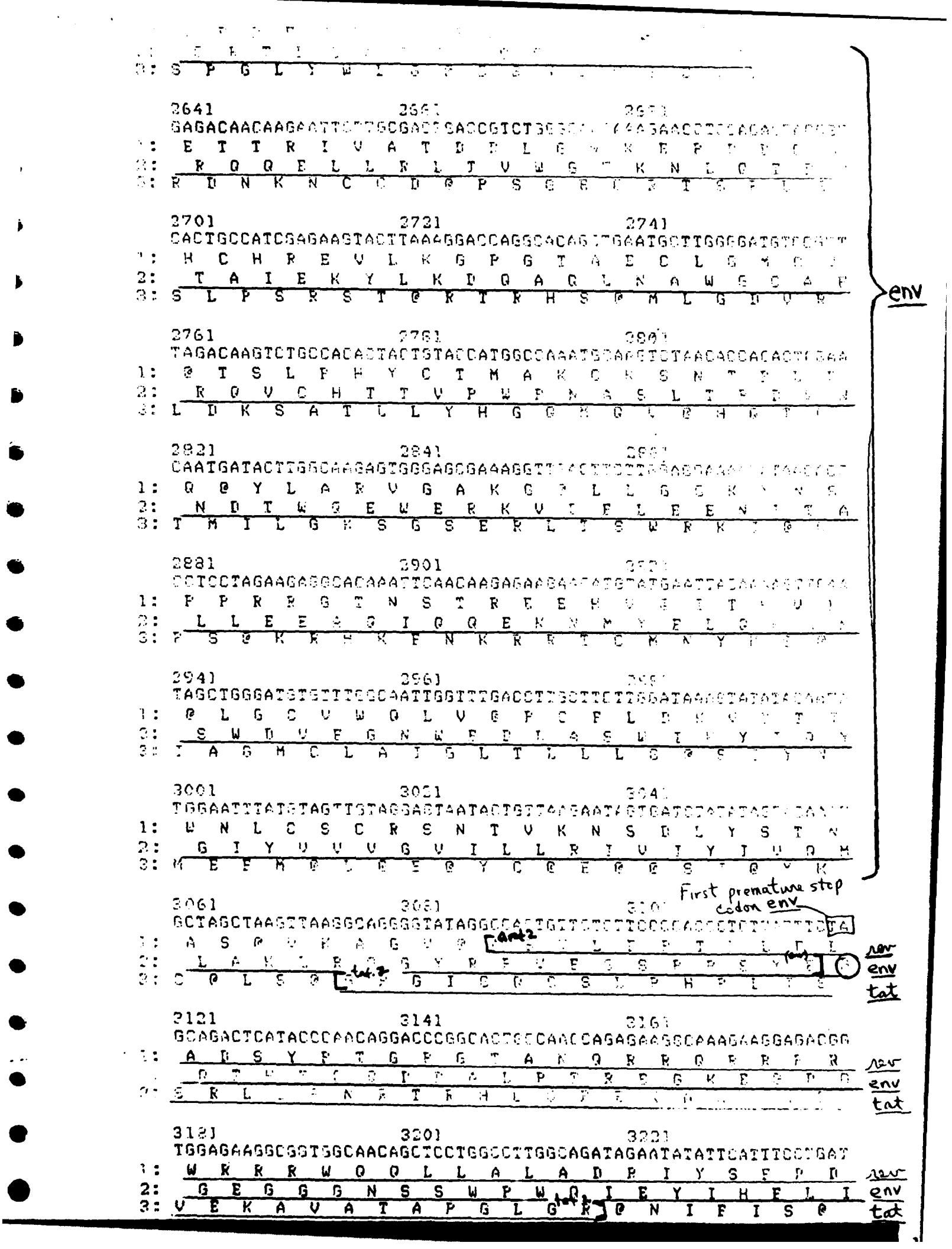
SIVmac251 FLB-10 3' sequence

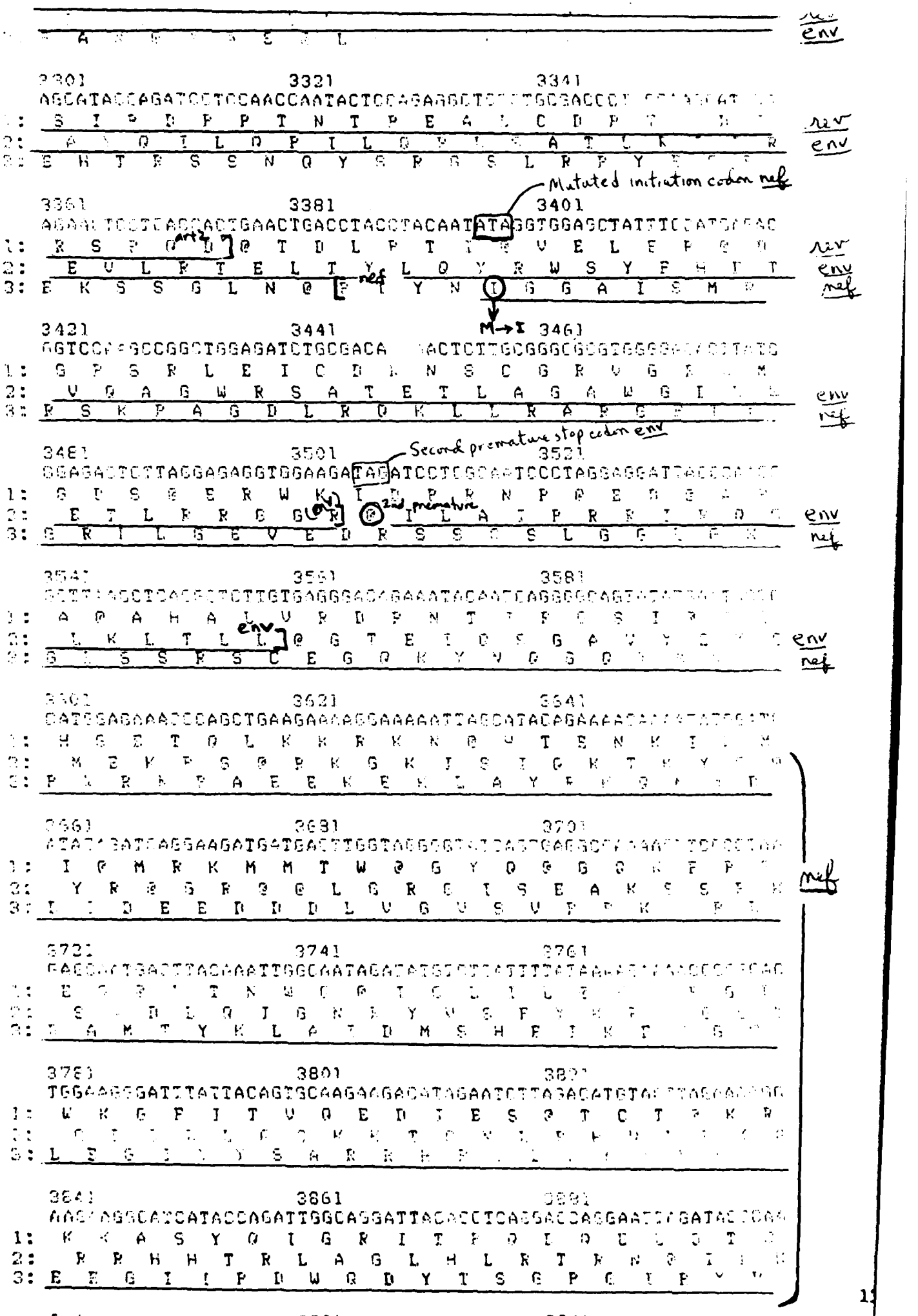


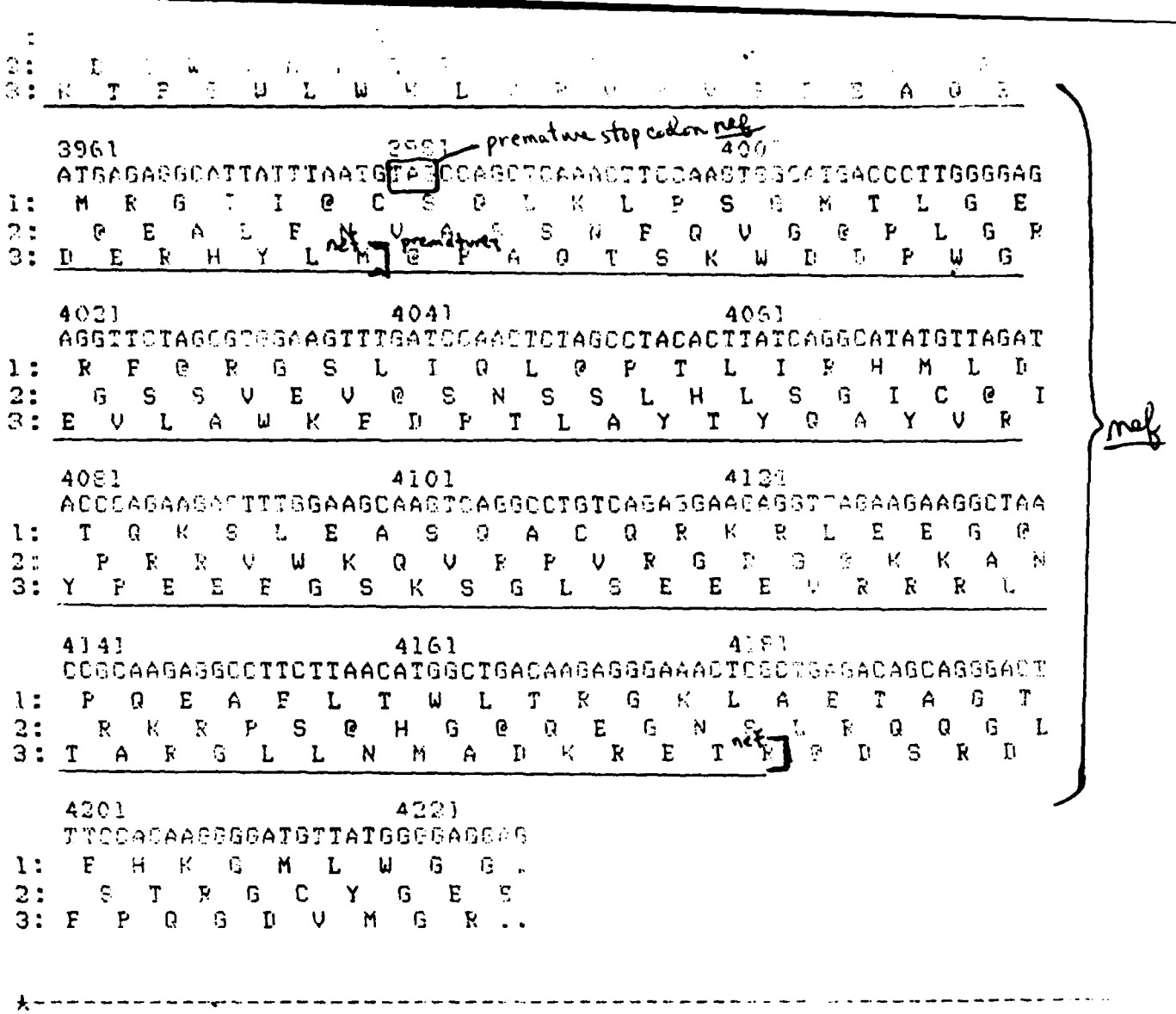

ATG
tat initiation codon
vpr
tat
env
rev
initiation codon rev
splice donor for rev and tat


env


env


env
First premature stop codon env
rev
env
tat
rev
env
tat
rev
env
tat


Mutated initiation codon nef
M→I
Second premature stop codon env
2nd premature
env
nef


premature stop codon
nef
nef
premature
nef